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Detection of mutations

FIELD OF INVENTION

The invention relates to mutations in the flavin-containing monooxygenase (FMO3) gene and methods for detection of such mutations in farm animals and poultry.

BACKGROUND OF INVENTION

Occurrence of fishy off-flavor in cow's milk

Fishy off-flavor in milk is a quality defect recently observed in bulk milk in Sweden, which causes considerable losses to both milk producers and dairy companies. Statistical data from an investigation made 1999 regarding the occurrence of off-flavor in milk showed that out of roughly 2,100 herds, 115 (i.e. > 5%) had received one or more complaints about fishy off-flavor in bulk milk. Altogether, these 115 herds had received 242 complaints about a moderate fishy off-flavor, whereas 18 bulk milk samples were classified as having a pronounced fishy odor/taste. Exact figures on the current prevalence of fishy odor among Swedish dairy cows are difficult to estimate as no routine organoleptic testing on milk from individual cows is performed. However, when random samples of bulk milk from individual farms were judged to have a fishy off-flavor, tests on samples from individual cows were sometimes performed. These tests have shown that the off-flavor most often originates from one or a few cows within a herd. In order to exceed the threshold above which the test panel perceives a fishy off-flavor in the farm bulk milk, a sufficiently high proportion of the total milk must come from affected cows. Consequently, an individual cow with a fishy off-flavor in the milk will go undetected unless she belongs to a fairly small herd. The above figure of 5% is thus likely an underestimate of the problem. To what extent this off-flavor occurs in other countries is not known, as the vast majority do not perform regular tests for off-flavor in bulk milk at the farm level. There are so far no indications that the defect has any fatal effects on traits subjected to natural selection, like calf survival, or traits included in the breeding goal for milk production.

Characteristics

Fishy off-flavor is characterized by a distinct, unpleasant taste and smell, reminiscent of rotting fish. A fishy smell in milk (Humphriss, 1953; Corfield, 1955) and coffee cream (Eyer *et al*. 1990) has previously been reported in connection with bacterial degradation of lecithin, choline, and betaine, the latter two being intermediate products in the breakdown of lecithin to trimethylamine (TMA) oxide with TMA as an intermediate compound. A

further potential source of fishy taint was related to selective oxidation of butterfat (Swoboda & Peers, 1977). The human nose is extremely sensitive to the TMA odor (Ayesh & Smith, 1990), e.g. the olfactory threshold for detection in milk lies around 1-2 ppm (Metha *et al.* 1974; von Gunten *et al.* 1976). As a consequence, milk from a few affected cows in a herd has been shown to be sufficient to cause a fishy off-flavor to the whole bulk milk. There have been a few reports on fishy odor in milk related to TMA content in milk from cows on wheat pasture (Metha *et al.* 1974; von Gunten *et al.* 1976; Kim *et al.* 1980).

Similar phenomena in other species

Problems with fishy odor associated with elevated TMA levels have been described in human ('Fish-odor syndrome' or 'Trimethylaminuria', OMIM #602079, http://www.ncbi.nlm.nih.gov) and chicken (Hobson-Frohock *et al.* 1973). In human, abnormal secretion of TMA has been observed in breath, urine, sweat, saliva, and vaginal secretions (Humbert *et al.* 1970), whereas in chicken the TMA has predominantly been found in egg yolk (Hobson-Frohock *et al.* 1973). The TMA is derived from the intestinal degradation of food/feed components rich in TMA or its precursors. Under normal conditions the TMA produced is oxidized to the odor- and tasteless compound TMA-oxide by the liver enzyme flavin-containing monooxygenase (FMO) (Hlavica & Kehl, 1977). The TMA oxide is thereafter excreted in the urine (Al Waiz *et al.* 1987*a*). The fishy odor is a consequence of impaired oxidation of TMA (Pearson *et al.* 1979; Spellacy *et al.* 1979).

Genetic causes

The fishy odor shows a recessive mode of inheritance in human (Al-Waiz *et al.* 1987*b*, 1987*c*; Ayesh *et al.* 1993) whereas in chicken it is described as 'semidominant' because the expression has been shown to be dependent on the ingestion of e.g. rapeseed meal (Bolton *et al.* 1976). In human, parents heterozygous for the defect were shown to excrete elevated amounts of TMA in the urine when given oral doses of 600 mg TMA (Al-Waiz *et al.* 1987*c*, 1989; Ayesh *et al.* 1990, 1993). However, in none of the cases did the treatment result in a fishy odor.

The fishy odor syndrome in humans has recently been shown to be due to mutations in *FMO3* encoding an isoform of flavin-containing monooxygenase (Dolphin *et al.* 1997*b*; Treacy *et al.* 1998; Akerman *et al.* 1999; Basarab *et al.* 1999; Forrest *et al.* 2001). The gene has been localized to chromosome 1q23-q25 (http://www.ncbi.nlm.nih.gov/LocusLink, August 2001) and its genomic sequence is known (Dolphin *et al.* 1997*a*). The human *FMO3* gene contains one non-coding and 8 coding exons and is 22.5 kb long. (GenBank AH006707, GenBank AL021026)

Dietary causes

The elevated concentration of TMA observed in various tissues in human, chicken, and cattle is likely to be due to a combination of genetic and dietary effects. Feed components rich in TMA precursors such as betaine, choline and sinapin, are beet products, green leaves and cereals, and rapeseed products. A high intake of these products may result in an accumulation of TMA, which in turn overloads the enzyme system. Rapeseed also contains progoitrin, a substance that in chicken has been shown to act as an FMO inhibitor by competing with TMA for the enzyme's active binding site (Pearson *et al.* 1982). In humans, the major source of TMA is marine fish and other seafoods (Zhang *et al.* 1999).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. (a) Alignment of codon 236-238 for the normal (R238) and mutant (R238X) *FMO3* allele in cattle. This region was analyzed using pyrosequencing. The location of the sequencing primer and the six positions sequenced are indicated. (b) Pyrosequencing results from the three different genotypes at codon 238. The sequenced reverse strand has the sequence (A/G)AGTGA where the A/G polymorphism corresponds to the R238X nonsense mutation.

DESCRIPTION OF THE INVENTION

According to the invention it is shown that the *FMO3* nonsense mutation R238X causes fishy off-flavor in milk. This is the first identified gene that has a profound influence on perceived quality of raw milk. The genotyping method described in this study can now be used by the breeding organisations to eliminate the problem in those breeds where a *FMO3* nonsense mutation is present.

The present invention provides a polypeptide which is a flavin-containing monooxygenase 3 (FMO3), wherein said FMO3 is a polypeptide comprising at least a sequence having at least 85% identity, preferably at least 90% identity, more preferably at least 95% identity, with the polypeptide sequence SEQ ID NO: 15.

The present invention further provides polypeptides which are flavin-containing monooxygenase 3 (FMO3), wherein said FMO3 is a polypeptide having at least 85% identity, preferably at least 90% identity, more preferably at least 95% identity, with the polypeptide sequence SEQ ID NO: 15.

An example of such a polypeptide is the bovine FMO3 as shown in SEQ ID NO:15. Further examples of polypeptides of the invention are the sheep and goat FMO3.

The present invention also provides polypeptides which are functionally altered mutants of flavin-containing monooxygenase 3 (FMO3), wherein said FMO3 is a polypeptide comprising at least a sequence having at least 85% identity, preferably at least 90% identity, more preferably 95% identity, with the polypeptide sequence SEQ ID NO: 15.

In the present context, an animal is considered to refer to all animal species, with the exception of humans.

In a preferred aspect, the present invention is directed towards mutants which result in buildup of TMA resulting in fishy taste and/or smell in an animal. Such mutants includes, but are not limited to, mutants of FMO3 with a decreased catalytic activity towards TMA oxidation.

An example of such a functionally altered mutant is a polypeptide resulting from a deletion of a part of the FMO3 polypeptide, such as the R238X variant of the bovine FMO3.

Other examples of polypeptides which are functionally altered mutants of flavin-containing monooxygenase 3 (FMO3) according to the invention, may arise from insertions, deletions, and mis-sense mutations in the *FMO3* gene.

The present invention further provides isolated nucleic acid sequences encoding flavin-containing monooxygenase 3 (FMO3), wherein said FMO3 is a polypeptide comprising at least a sequence having at least 85% identity, preferably at least 90% identity, more preferably at least 95% identity, with the polypeptide sequence SEQ ID NO: 15.

Examples of such isolated nucleic acids are the cDNA sequence encoding the bovine FMO3 shown in SEQ ID NO: 14, the bovine *FMO3* gene, or fragments thereof. Examples of such fragments are exons 3, 6, 7 and 9 shown as SEQ ID Nos: 9, 10, 11, and 12, respectively.

Nucleic acid sequences of the invention include variants of the *FMO3* gene, such as the R238X and E287G variants of the bovine *FMO3* gene.

To determine the percent identity of two amino acid sequences or of two nucleic acids sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid

residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity= # of identical positions/total # of positions (e.g., overlapping positions) x 100). In one embodiment the two sequences are the same length.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad Sci. USA 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecule of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25:3389. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

The invention also includes specific fragments of a nucleic acid sequence of the invention. "Specific fragment" refers to a nucleic acids fragment having a sequence that is found only in the nucleic acid sequences of the invention, and is not found in nucleic acid sequences encoding related polypeptides such as FMO1, FMO2, FMO4, and FMO5. Such fragments may be, but are not limited to, primers used in amplification reactions such as PCR, or in hybridization experiments. Such fragments are therefore typically at least 10 bp in length, such as at least 15 bp, more preferably at least 20 bp, but may also be longer, such as at least 50 bp or at least 100 bp. The degree of identity between the different FMOs seems to be the same over the whole sequence, the degree of identity between FMO3 and FMO2 (which is the closest relative) being around 60%. The degree of identity between e.g. bovine and human FMO3 is roughly the same over the whole sequence.

"Specifically hybridising fragments" refers to nucleic acid fragments which can hybridize, under stringent conditions only with nucleic acids of the invention, without hybridizing with nucleic acid sequences encoding related polypeptides such as FMO1, FMO2, FMO4, and FMO5.

Said specific fragments or specifically hybridising fragments may for example be used as primers or probes for detecting and/or amplifying nucleic acid sequences encoding FMO3 polypeptides.

Defining appropriate hybridization conditions, including stringent conditions, is within the skill of the art. See, e.g., Molecular Cloning: A Laboratory Manual, 3rd ed., Sambrook et al. eds., Cold Spring Harbor Laboratory Press, 2001; DNA Cloning: A practical Approach, Glover & Hames eds., Oxford University Press, 1996; Nucleic Acid Hybridization: Essential techniques, Ross ed. Wiley, 1998. Thus, stringent hybridization conditions are chosen, by varying the temperature and/or salt concentration in hybridization experiments, such that principally only the molecule of interest hybridizes to the target sequence.

The invention includes sets of primers comprising at least one primer consisting of a specific fragment or specifically hybridising fragment as defined above.

As shown herein, mutations in the bovine *FMO3* gene may lead to fish off- flavour of milk, caused by an altered metabolism of trimethylamine.

It is further postulated that mutations in the chicken *FMO3* gene may be associated with fish off-flavour of eggs produced by hens carrying such mutations.

The invention also includes a method for detecting a mutation in the *FMO3* gene of an animal where the mutation will cause an alteration in the metabolism of trimethylamine leading to a fish off- flavour in a food product of the animal or its offspring, the food product can be any food product collected or produced from animals or their offspring, such as milk or eggs. The animal can be a mammal, excluding humans, used in the production of such food products, such as a cow, a bull, a sheep, or a goat. The animal may also be poultry, such as a hen or a cock.
The method comprises:

- obtaining a nucleic acid sample from the animal;
- determining the presence in said nucleic acid sample of a nucleic acid sequence encoding a mutated FMO3.

In yet another embodiment the invention provides a method for detecting a nucleic acid sequence comprising a mutation in the *FMO3* gene of an animal where the mutation will cause an alteration in the metabolism of trimethylamine leading to a fish off-flavour in a food product of the animal or its offspring.

The method comprises:

- obtaining a nucleic acid sample from the animal;
- contacting said nucleic acid sample with a nucleic acid probe spanning said mutation under conditions of specific hybridisation between said probe and the mutant sequence to be detected
- detecting the hybridisation complex.

Preferably, the process of the invention further comprises, prior to hybridization, PCR amplification from the nucleic acid sample, of a sequence comprising at least the portion of the *FMO3* sequence wherein the mutation is to be detected.

Methods allowing the specific hybridisation of a probe only with a perfectly matching complementary sequence, and useful for the detection of punctual mutations are known in the art. They include for instance Allele Specific PCR (Gibbs , Nucleic Acid Res. 17: 2427-2448, 1989), Allele Specific Oligonucleotide Screening (Saiki et al. Nature 324:163-166, 1989).

A mutation in the *FMO3* gene may also be detected through detection of polymorphic markers closely linked to said mutation. Such polymorphic markers may be used as surrogate markers for the functional mutation itself, provided that they are in linkage disequilibirum with the mutation. In general, the closer the polymorphic marker is to the mutation itself, the greater the likelyhood that the marker may be used as surrogate marker. However, due to variations in recombination frequencies throughout the genome, the physical distance at which the surrogate marker may be from the mutation is likely to be variable. The invention also provides means for identifying said polymorphic markers, and more specifically polymorphic markers comprised within a genomic DNA sequence comprising at least a portion of a *FMO3* gene, and up to 500 kb, preferably 300 kb, more preferably up to 100 kb of a 3' and/or of a 5' adjacent sequence, or the complement thereof In table 3, examples of polymorphic markers identified in cDNA sequences from normal alleles from a carrier of the R238X mutation and from a homozygous normal individual are provided.

Said polymorphic markers may be obtained for instance, by screening a genomic DNA library from an animal with a probe specific for the *FMO3* gene, in order to select clones comprising said nucleic acid sequence and flanking genomic sequences, and identifying a

polymorphic marker in said flanking genomic sequences. The allele(s) of a polymorphic marker associated with a given mutant allele of the *FMO3* gene may also easily be identified by use of a genomic DNA library from an individual wherein the presence of said mutant allele has previously been detected by hybridisation with a nucleic acid probe of the invention.

Polymorphic markers include for instance, single nucleotide polymorphisms (SNP), microsatellites, insertion/deletion polymorphisms and restriction fragment length polymorphisms (RFLP). These polymorphic markers may be identified by comparison of sequences flanking the *FMO3* gene obtained from several individuals. Microsatellites may also be identified by hybridisation with a nucleic acid probe specific of known microsatellite motifs using techniques known to those skilled in the art. Once a polymorphic marker has been identified, a DNA segment spanning the polymorphic locus may be sequenced and a set of primers allowing amplification of said DNA segment may be designed. The invention also encompasses said DNA primers. Design and use of DNA primers for the purpose of amplification by PCR or hybridization is within the skill of the person of ordinary skill.
Thus, appropriate primers can be selected from the sequences of the invention using methods known to those skilled in the art.

Detection of a mutation in *FMO3* gene may be performed by obtaining a sample of genomic DNA from an animal, amplifying a segment of said DNA spanning a polymorphic marker by PCR using a set of primers of the invention, and detecting in said amplified DNA the presence of an allele of said polymorphic marker associated with said mutation.

The invention also provides kits for the practice of the methods of the invention. A kit comprises at least one specific fragment of a nucleic acid of the invention, or at least one nucleic acid fragment able to hybridise with a nucleic acid sequence of the invention. Said nucleic acid may be labelled using techniques known to those skilled in the art. Examples of suitable labels include, but are not limited to, radioactive labels, fluorescent labels and affinity labels such as biotin. The kits may also comprise a set of primers of the invention. They may be used in conjunction with commercially available amplification kits. They may also include positive and/or negative control reactions or markers, molecular weight size markers for gel electrophoresis, and the like.

A preferred embodiment of the invention relates to the identified R238X mutation in the bovine FMO3 and methods to identify the mutation in order to be able to remove that mutation from stock. The genotyping methods of the present invention can be used to eliminate the problem in those breeds where a *FMO3* nonsense mutation, or other mutations leading to fishy odor or taste, is present. A practical way to reduce the problem

would be to genotype currently used breeding bulls, bull dams, and young potential breeding bulls before progeny testing, determine which animals are carriers of the mutation(s), and eliminate carriers from breeding. That way, the detrimental mutation(s) can be eliminated from the selected breed.

Realization of the invention can be illustrated by the following examples. These examples should however only be understood as examples of specific embodiments of the present invention, and in no way limiting for its adaptation for other use.

EXAMPLES

Example 1. Sequencing of the bovine FMO3 gene

Cattle samples and DNA preparation

The present study included two different groups of dairy cattle. The first material comprised 21 cows of the Swedish Red and White breed (SRB) with information from sensory analyses on the milk. Ten of the cows belong to either of the two experimental herds, Jälla and Kungsängen, at Swedish University of Agricultural Sciences in Uppsala. The remaining cows belong to either of four commercial herds, all of which had received complaints about fishy off-flavor in the bulk milk. In three of the six herds, 'control' cows were chosen that were comparable to the affected cows as regards breed, lactation number, and stage of lactation but were producing milk with a normal taste and smell.

A population study was performed in which bulls and cows from the four dairy breeds in Sweden were genotyped for the observed *FMO3* nonsense mutation. From each of the two major breeds, SRB and Swedish Holstein, 100 individuals were chosen, whereas from the two small breeds, Swedish Polled and Swedish Jersey, 25 and 23 individuals were sampled, respectively. Genomic DNA was prepared from blood samples according to standard protocols.

PCR amplification and sequencing of the bovine FMO3 gene from genomic DNA samples

Exonic parts of the *FMO3* gene were amplified from genomic DNA samples using primers corresponding to sequences well conserved between the human, rabbit, mouse, and rat *FMO3* sequences available in GenBank (Table 1). Two genomic DNA samples were used, one from a cow that had been shown to repeatedly produce milk with a fishy flavor and the other from a control cow producing normal milk.

Table 1. Primer sequences used for PCR amplification of the bovine *FMO3* gene from genomic DNA.

FMO3 region amplified[1]	Forward (F) and reverse (R) primer sequences 5'-3'		
exon 3	*F:*	*GACCATGCAGAAGAGGGCAG*	*SEQ ID NO: 1*
	R:	CTTAAACTGTATGTATTTCAGGAGGTT	SEQ ID NO: 2
exon 6	*F:*	*CATCATCAGCTCCAGAAGTGG*	*SEQ ID NO: 3*
	R:	TAAAGGCATCAAGCCATAGTT	SEQ ID NO: 4
exon 7	F:	CAGAATCCTGAGGAAAGAGCC	SEQ ID NO: 5
	R:	ATTACTTGTGCTGCCCAGCG	SEQ ID NO: 6
exon 8-9	F:	GATGAATGATATTAATGAGAAAATGG	SEQ ID NO: 7
	R:	CCGGTCCCACTGGGTCAG	SEQ ID NO: 8

[1]The numbering of exons is based on the described exon-intron organization for the human *FMO3* gene (Dolphin *et al*. 1997a).

The PCR was performed in 10 µl reactions including 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2.5 pmol of each primer, 25 ng genomic DNA, and 0.35 U Ampli*Taq* DNA polymerase (Perkin Elmer, Branchburg, NJ, USA). Thermocycling was carried out in a PTC200 machine (MJ Research, Watertown, MA, USA) and included 40 cycles with annealing at 53°C for 30 s and extension at 72°C for 2 min. The denaturation step was 95°C for 1-2 min in the first two cycles, and 94°C for 30 s in the remaining cycles. The products were analyzed by agarose gel electrophoresis (4% Nusieve/Seakem 3:1, FMC Bioproducts, Rockland, ME, USA). The PCR products were directly sequenced with BigDye terminators and an ABI377 instrument (Perkin-Elmer, Foster City, CA, USA). Sequence analysis and comparison of sequences from the cows with fishy flavored and normal milk, respectively, was performed using the Sequencher software (GENE CODES, Ann Arbor, MI, USA).

Bovine *FMO3* gene sequences

Bovine *FMO3* sequences corresponding to human *FMO3* exons 3, 6, 7, 9, and intron 8 were generated and are given in SEQ ID Nos: 9-13. Subsequently, the complete coding region of the bovine *FMO3* gene was sequenced from RT-PCR products from liver mRNA. The bovine *FMO3* cDNA sequence is given in SEQ ID NO: 14 and the corresponding polypeptide

sequence of bovine FMO3 is given in SEQ ID NO: 15. The exonic sequences generated from genomic DNA showed 100% identity to the cDNA sequence obtained from the RT-PCR products and *FMO3* specificity was strongly supported by the high sequence similarity to other mammalian *FMO3* sequences. For example, a Blast comparison between the complete coding cattle *FMO3* sequence obtained here and the corresponding human sequence (GenBank NM_006894) showed 85% and 82% identity at the nucleotide and amino acid levels, respectively.

Identification of a nonsense mutation in the bovine FMO3 *gene*

Partial cattle *FMO3* sequences obtained from a cow producing milk with strong fishy off-flavor and a cow producing normal milk were compared. The compared sequences included altogether 1522 bp and 808 bp of these represent coding sequence. The coding part includes 268 codons, corresponding to approximately 50% of the protein. The only sequence difference found between the two cows was a C/T nucleotide substitution located at position 62 in exon 6 (SEQ ID NO: 10). The C→T substitution changes the codon for arginine (R) at position 238 to a stop codon (X); the numbering of amino acid positions are based on the human amino acid sequence given in Genbank NM_006894. This nonsense mutation is thus denoted R238X. The pyrosequencing test confirmed that the affected cow was homozygous for the R238X substitution whereas the control cow was homozygous R/R at residue 238 (Fig. 1).

<u>*Example 2. Association between mutations in FMO3 and the presence of fishy of-flavour in cow's milk.*</u>

Genotyping of the *FMO3* R238X nonsense mutation

The identified single nucleotide polymorphism (SNP) in exon 6 was genotyped by pyrosequencing (Ronaghi et al. 1998) using a Luc96 instrument (Pyrosequencing AB, Uppsala, Sweden). This included PCR amplification of a 147 bp product from genomic DNA samples using the forward primer 5'-biotin- GAT GAA GGC TAT CCA TGG GAC (SEQ ID NO: 16) and the reverse primer 5'-TAA AGG CAT CAA GCC GTA GTT CTC (SEQ ID NO: 17). The PCR was performed in 20 μl reactions with concentrations of reagents as above. Thermocycling was carried out in a PE9600 instrument (Perkin-Elmer, Foster City, CA, USA) and included an initial denaturation step at 94 °C for 2 min followed by 5 cycles with 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, 43 cycles with 94°C for 20 s, 50°C for 30 s, 72°C for 45 s, and a final extension step at 72 °C for 5 min. Pyrosequencing on the forward strand was done using the reverse sequencing primer 5' TGA GGA ATG TTT CAA ATC (SEQ ID NO: 18) and conditions according to the manufacturer's recommendations.

RT-PCR amplification and sequencing of the bovine FMO3 *cDNA*

Ten livers from SRB cattle were collected and the pyrosequencing test showed that two were heterozygous carriers of the R238X mutation. One of these livers as well as one liver from a homozygous wild-type cow were used for mRNA preparation, reverse transcription (RT)-PCR amplification and direct sequencing of the coding FMO3 region. Two additional primers were used for this purpose, designed from sequences in the untranslated regions which showed conservation between other mammalian FMO3 sequences. Thus, the 5'UTR primer 5' GGA CTT AGA CAC ACA GAA GAA AAG AAG (SEQ ID NO: 19) and the 3'UTR primer 5' GAG GTG TGA AAT TCT TAT TTT TTA AAT AG (SEQ ID NO: 20) were used in pairs with the reverse and forward pyrosequencing PCR primers, respectively. Thus, the coding sequence was amplified in two overlapping pieces, each including the site of the R238X mutation. Poly A mRNA was isolated from cattle livers using the Quickprep mRNA Micro purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and RT-PCR was carried out using the First strand cDNA synthesis kit (Amersham Pharmacia Biotech) with ~200 ng mRNA primed with 0.1 μg random hexamers in a 15 μl reaction volume. Two μl of the completed first strand reaction was used in each PCR reaction with a total volume of 12 μl so that the final concentrations of dNTP, Mg^{2+}, primers, and AmpliTaq polymerase were the same as above. The thermocycling conditions were as described above for the sequenced genomic DNA amplicons but the 5' part of the cDNA required a repeated PCR to yield enough product for sequencing.

The R238X substitution is closely associated with the presence of fishy off-flavor in cow's milk

The pyrosequencing test was used to test a case-control material comprising animals producing milk classified as having a fishy off-flavor and control animals from the same herds (Table 2). A Class 2 score means that two trained persons both classified the sample as having a strong fishy off-flavor whereas Class 1 implies that the two persons judged the fishy off-flavor as strong/moderate, moderate/moderate or moderate/normal. The results revealed a very strong association between the *FMO3* R238X nonsense mutation and strong fishy-off flavor since eight out of nine animals with the Class 2 score were homozygous for this mutation and the ninth was heterozygous. In contrast, the five control samples were homozygous normal. The Class 1 group contained animals of all three genotype classes.

Table 2. *FMO3* genotype distributions in a group of 21 Swedish Red and White cows with or without fishy off-flavor in milk and in animals representing the four different dairy breeds in Sweden

	FMO3 genotype at codon 238			
	R/R	R/X	X/X	Total
Case/control study				
Sensory evaluation:[1]				
Normal	5	0	0	5
Class 1	3	3	1	7
Class 2	0	1	8	9
Population study				
Breed:				
Swedish Red and White	71	27	2	100
Swedish Holstein	100	0	0	100
Swedish Polled	25	0	0	25
Swedish Jersey	23	0	0	23

[1]Evaluation based on two trained persons. Class 2 = both person recognized a strong off-flavor (strong/strong); Class 1 = strong/moderate, moderate/moderate, moderate/normal.

The R238X substitution occurs at a surprisingly high frequency in the Swedish Red and White breed

The population screening involved 248 animals representing the four dairy breeds in Sweden. The R238X substitution was not found in Swedish Holstein, Swedish Polled, or Swedish Jersey but was surprisingly common in the Swedish Red and White breed (Table 2). Two homozygous mutant and 27 heterozygotes were observed among 100 animals and the allele frequency estimate for the mutation was as high as 15.5%.

SNPs observed by cDNA sequencing of a heterozygous R238X carrier and a homozygous normal animal

Only the wild type transcript appeared well represented in the mRNA sample from the R238X carrier. Only a very weak sequencing signal could be observed from the mutant transcript, at least 10 times weaker than from the wild type transcript. A similar result was

obtained using liver mRNA from a second heterozygous R238X carrier. Consequently, we could not search for possible additional mutations present in the *FMO3* allele associated with fishy-off flavour. However, the RT-PCR experiment confirmed that the R238X mutation occurs in an expressed *FMO3* gene.

The comparison of the full-length liver cDNA sequence from the normal allele from the carrier and from the homozygous normal individual revealed three additional single nucleotide polymorphisms (SNPs), all compiled in Table 3. Only one of the SNPs altered the amino acid sequence, E287G.

Table 3. Single nucleotide polymorphisms in the bovine *FMO3* gene

Nucleotide/Amino acid position[1]	Nucleotide/Amino acid change
877/287	A↔G / E↔G
1067/350	T↔C / no change
1085/356	A↔C / no change

[1]Position as numbered in SEQ ID NO: 14.

Discussion

The present study has provided compelling evidence for that the observed *FMO3* nonsense mutation R238X causes fishy off-flavor in milk. This conclusion is based on (i) the identification of an obvious loss-of-function mutation in a gene associated with a similar syndrome in humans, (ii) a very strong association between the presence of this mutation and a strong fishy-off flavor in a case/control material and finally (iii) the presence of this mutation in the only breed (SRB) tested with well documented cases of a fishy off-flavor.

The R238X mutation causes a premature termination eliminating more than 50% of the FMO3 enzyme which is expected to comprise 532 amino acids as in the human FMO3 protein. Similar nonsense mutations in humans (E305X, E314X) have been shown to lead to a complete loss of enzyme activity (Treacy *et al.* 1998, Akerman *et al.* 1999). Moreover, we observed only barely detectable levels of the mutant transcript in liver mRNA from two heterozygous carriers of R238X. This is most probably explained by nonsense-mediated mRNA decay (NMD; Culbertson 1999). It is very likely that cows homozygous for the R238X mutation show a complete lack of FMO3 activity necessary for the oxidation of TMA to an odorless compound. This provides a plausible explanation for the fishy off-flavor of the milk from these cows. Milk samples from five of the cows homozygous for the mutation and included in this study have previously been analyzed for TMA content using dynamic

headspace gas chromatography. The results showed that these milk samples all had comparatively high concentrations of TMA whereas the milk from normal cows showed non-detectable or, in a few cases, very low TMA concentrations.

The results suggested a recessive mode of inheritance for the fishy-off flavor in cows milk in agreement with the recessive inheritance of the fishy-odor syndrome in humans. However, there was not a perfect agreement between this model and the observations from the case/control study since one carrier was scored as having strong fishy off-flavor (Class 2) and one homozygous mutant showed a moderate off-flavor (Class 1). We think there are two potential explanations from this deviation from a strict recessive model. The fishy-off flavor is influenced by environmental factors, like the presence of TMA precursors or FMO inhibitors in the feed. For example, the TMA concentration in the milk from one of the homozygous mutants was shown to vary between 1 and 16 mg TMA/kg milk within a single lactation. Secondly, the sensory evaluation is a somewhat subjective measure. The test panel consisting of two trained persons goes through a considerable number of milk samples during one day and may have some difficulties to discern the various off-flavors present in the milk samples and also to discriminate between different flavors.

In the present material the mutation was only observed among the SRB animals, but at a relatively high frequency. This is in complete accordance with the experiences made by the staff at the milk analysis laboratory that performed the sensory analysis, who have so far only encountered the fishy off-flavor in milk from SRB cows. We analyzed the pedigree data for all carriers and homozygous mutants but we did not find any common ancestor of these animals within the last 10 generations. The results suggest that the mutation has been present in the existing SRB population for a relatively long period of time. The origin of the mutation probably dates back at least to the beginning of the last century, judging from the available pedigree information on affected animals. Moreover, the pedigree data indicate that the mutation may exist in other cattle populations closely related to the Ayrshire.

To the best of our knowledge, this is the first identified gene that has a profound influence on perceived quality of raw milk. The genotyping method described in this study can now be used by the breeding organisations to eliminate the problem in those breeds where a *FMO3* nonsense mutation is present. A practical way to reduce the problem would be to genotype currently used breeding bulls, bull dams, and young potential breeding bulls before progeny testing and eliminate carriers from breeding.

Example 3. Sequencing of the chicken *FMO3* gene

Exonic parts of the chicken *FMO3* gene will be amplified from genomic DNA samples using both primers corresponding to sequences well conserved between the bovine, human,

rabbit, mouse, and rat FMO3 sequences as well as primers corresponding to EST sequences derived from the chicken *FMO3* gene, ESTs 603149708F1 and 603610105F1 available in the Chicken EST database (http://www.chick.umist.ac.uk/cgi-bin/chicken_database.cgi). Both genomic DNA samples from hens that have been shown to produce eggs with a fishy off-flavor and genomic DNA samples from normal hens will be used as templates. The sequence of cDNA coding for the chicken FMO3 will be obtained from RT-PCR products from liver mRNA.
The sequences obtained from hens that have been shown to produce eggs with a fishy off-flavor and sequences obtained from normal hens will be compared and sequence variants identified.

Hens will be genotyped and *FMO3* variants associated with production of eggs with fishy off-flavour will be identified.

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